J. Plant Biol. (2010) 53:444–452
DOI 10.1007/s12374-010-9135-6

ORIGINAL RESEARCH

Overexpression of *OsVP1* and *OsNHX1* Increases Tolerance to Drought and Salinity in Rice

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Received: 18 July 2010 / Revised: 25 October 2010 / Accepted: 26 October 2010 / Published online: 11 November 2010

Abstract Drought and salinity are major abiotic stresses affecting rice production. To improve plant tolerance to salinity and drought, we overexpressed rice Na^+/H^+ exchangers (OsNHX1) and H^+-pyrophosphatase in tonoplasts (OsVP1) in a japonica elite rice cultivar, Zhonghua 11. Compared with our wild-type control, transgenic plants overexpressing both genes incurred less damage when exposed to long-term treatment with 100 mM NaCl or water deprivation. Under high-saline conditions, the transformants accumulated less Na^+ and malondialdehyde in the leaves, thereby allowing the plants to maintain a low level of leaf water potential and reduce stress-induced damage. Those transgenics also had higher photosynthetic activity during the stress period. Under those conditions, they also showed an increase in root biomass, which enabled more water uptake. These results suggest that *OsVP1* and *OsNHX1* improve the tolerance of rice crops against drought and salt by employing multiple strategies in addition to osmotic regulation.

Keywords Drought tolerance · H^+- pyrophosphatase · Osmotic potential · Salt tolerance · Tonoplast · Transgenic rice (*Oryza sativa*) · Vacuolar Na^+/H^+ exchanger

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Abbreviations

DO	Double overexpression
NHX1	Na^+/H^+ exchanger 1
RWC	Relative water content
WT	Wild type
VP (V-PPase)	Vacuolar H^+-pyrophosphatase

Drought and soil salinization have major impacts on crop growth and development, limiting agricultural productivity worldwide. Nearly half of all irrigated land is affected by salinity (Munns 2002; Flowers 2004). Rice, the most important food cereal, grows under standing water conditions. Thus, salt and drought stresses can severely influence its yield (Qian et al. 2003; Hu et al. 2006). Rice cultivars with improved stress tolerance can be produced by genetically manipulating the expression of one or more genes (Apse et al. 1999; Gao et al. 2003; Shi et al. 2003; Zhang et al. 2004; Sreenivasulu et al. 2007). In fact, pyramiding multiple genes into one genotype can achieve a greater degree of stress tolerance compared with the utilization of a single gene (Muehlbauer et al. 2006).

A high salt content increases osmotic stress (Gaxiola et al. 2001; Mohanty et al. 2002) and leads to the accumulation of excess sodium in plants (Niu et al. 1995; Zhu 2001). To reduce the damage from such stress, plants can employ several defense responses, e.g., maintaining cellular ion homeostasis (Hunter et al. 2007). Vacuolar H^+-pyrophosphatase (H^+-PPase, VP) and Na^+/H^+ antiporters (NHX), located in the tonoplasts, are key elements in the osmoregulation system, compartmentalizing Na^+ into the vacuoles and reducing the Na^+ concentration in the cytoplasm (Gaxiola et al. 1999; Wang et al. 2001). H^+-PPase pumps H^+ from the cytosol into the vacuole, a process coupled to the hydroxylation of PPi (Gaxiola et al.

2001; Brini et al. 2007). The electrochemical potential gradient of H^+ between the cytoplasm and vacuole can promote the exchange of Na^+/H^+ through antiporters and enable the transport of some small organic acids and sugars (Zhao et al. 2006). These antiporters move sodium ions from the cytoplasm into vacuoles in exchange for protons across membranes, thereby reducing the toxic effects of excessive Na^+ in the cells (Barklaz et al. 1995; Gaxiola et al. 2001; Fukuda et al. 2004).

Vacuolar NHX and VP are involved in salt and drought tolerances within plants (Gaxiola et al. 2001; Zhang et al. 2001; Ohta et al. 2002; Apse et al. 2003; Fukuda et al. 2004; Ma et al. 2004). Overexpression of a vacuolar Na^+/H^+ antiporter or H^+ pump (H^+-pyrophosphatase) increases such tolerance in transgenic plants (Apse et al. 1999; Gaxiola et al. 2001; Zhang and Blumwald 2001; Xue et al. 2004). For example, those that overexpress *AtNHX1* accumulate Na^+/H^+ antiporters in their tonoplasts and show improved tolerance to NaCl (Apse et al. 1999; Zhang and Blumwald 2001). The degree of tolerance is positively correlated with the abundance of *AtNHX1* transcripts. Furthermore, overexpression of a vacuolar H^+-PPase in *Arabidopsis thaliana* enhances salt and drought tolerances (Gaxiola et al. 2001; Park et al. 2005) and is associated with greater cell division and auxin transport (Li et al. 2005). Co-expression of *SsNHX1* and *AVP1* in rice also confers higher salt tolerance than that achieved by single-gene transformation (Zhao et al. 2006).

To investigate the role of native VP and NHX genes in establishing tolerance to salt or drought in rice, we overexpressed two tonoplast genes: H^+-PPase (OsVP1) and the Na^+/H^+ antiporter (OsNHX1). Plants engineered for overexpression of both genes (the double overexpression line) were compared with a wild-type (WT) control as well as with transgenic lines that overexpressed only *OsVP1* or *OsNHX1*.

Materials and Methods

Plasmid Constructs and Rice Transformation

cDNA fragments with full ORFs of *OsVP1* and *OsNHX1* were amplified and inserted into a pTF102-derived binary vector (Frame et al. 2002). The resultant vectors contained the transgene driven by the cauliflower mosaic virus 35S promoter and a selectable marker gene (BAR) cassette. These vectors were transferred into *Agrobacterium tumefaciens* strain EHA105 via electroporation for transforming rice (*Oryza sativa*).

Mature and sterile seeds of "Zhonghua 11" (a japonica cultivar) were used for inducing calli, which were then co-cultivated with *A. tumefaciens* containing the gene(s) of interest. Transformed calli were transferred to a selection medium (N6+250 $\mu g mL^{-1}$ carbenicillin+2.5 $\mu g mL^{-1}$ Bialophos herbicide, or Basta) for regeneration.

RT-PCR Analysis

Total RNA was extracted from 200 mg of young rice leaves using TRIzol reagent (Invitrogen, USA). RNA samples (5 μg) were reverse-transcribed with M-MLV reverse transcriptase (Promega, USA) and an oligo(dT)18 according to the manufacturer's protocol. PCR was conducted with 2 μL of template cDNAs and two pairs of gene-specific primers that spanned the introns: 5′-CCTGGAGACAGCAAGTTGT-3′ and 5′-CTCTGCTCGGTTGGTGATC-3′ for OsNHX1, and 5′-AAGATGACCCAAGAAACCCA-3′ and 5′-GGTACAG CATAGGAGTGAAT-3′ for OsVP1. For our internal control, a fragment of *OsActin1* (accession no. X63860) was amplified with primers 5′-CTGACGGAGCGTGGTTACTCAT-3′ and 5′-TGGTCTTGGCAGTCTCCATTTC-3′. PCR conditions included 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; then a final extension at 72°C for 5 min. Real-time qRT-PCR was performed with a SYBR Premix Ex TaqTM (Perfect Real Time) Kit (TaKaRa Biomedicals, Tokyo, Japan) on a Light-Cycler480 machine (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Crossing and Verification of Double Transformants

The T_3-generation homozygotes of *OsVP1* and *OsNHX1* were crossed, with the *OsVP1* transgenic plants serving as the female parent. A PCR-based approach was taken to identify double overexpression (DO) plants in the F_2 population. *OsVP1*-specific primers spanned a 338-bp fragment in the genomic DNA and 176 bp in the cDNA. This difference was used to distinguish between WT and transgenic plants. Identification of *OsNHX1* was performed with an upper primer of CaMV 35S (5′-ACGTAAGGGAT GACGCACA-3′) and a lower primer (5′-GGACTCATT GACCCAGCGATTCT-3′) within *OsNHX1*.

Salt and Drought Treatments

WT and transgenic seeds were germinated at 37°C and then transferred to a hydroponic solution in the greenhouse (60% humidity, 35,000 lx light intensity, and a 12-h photoperiod). After 2 weeks of growth in soil-filled pots, the seedlings were exposed to saline stress for another 3 weeks by watering them with a fresh 100 mM NaCl solution every 3 days. For drought treatment, the initial soil water capacity was recorded as described by Shou et al. (2004). Pots containing the WT control and transgenic plants were then watered to achieve 20%, 30%, 40%, 50%, or 100% of the

maximum water capacity (MWC, defined as the total amount of water stored in the soil). To standardize the experimental conditions, 20% MWC (determined as the wilting point for WT plants) was used for evaluating drought tolerance.

Na^+ Content in Leaves

Leaves were harvested at 15 days after the salt treatment began and were rinsed twice with distilled water. After drying at 120°C, their dry weights (DW) were determined. The tissues were then burned to ash at 450°C overnight and dissolved in 1:1 HNO_3. Sodium content was measured by atomic absorption spectrophotometry as described by the manufacturer (AA-650, Shimadzu Corporation, Japan). The data were statistically analyzed with a Student's *t* test.

Relative Water Content

For calculating relative water content (RWC), leaves at the same position on each plant were collected after treatment and weighed immediately (for salt treatment, this was performed on day 7, when the non-transformants had died). After floating in deionized water at 4°C overnight, the rehydrated leaves were weighed then dried overnight at 70°C to determine DW. Relative water content was computed as:

$$\mathrm{RWC} = (\text{fresh weight} - \text{dry weight})/(\text{rehydrated weight} - \text{dry weight}).$$

Net Photosynthetic Rate (P_n) and Leaf Water Potential

After the salt and drought treatments were concluded, P_n values were recorded from the leaves with an automatic photosynthetic measuring apparatus (LI-6400, LiCor, USA). Three repeats were designed for each line, with each repeat containing six plants. Leaf water potential was monitored by a PsyPro vapor pressure psychrometer (Wescor, USA).

MDA and Proline Contents

Malondialdehyde (MDA) and proline contents were assayed according to a protocol previously described by Peever and Higgins (1989). Three hundred micrograms of leaf tissue was used for MDA and 500 μg for proline. The concentration of proline ($\mu mol L^{-1}$) was spectrophotometrically determined by the absorbance at a wavelength of 520 nm. Absorbance at 450, 532, and 600 nm was used to determine the concentration ($\mu mol L^{-1}$) of MDA:

$$C = 6.45(A_{532} - A_{600}) - 0.56(A_{450})$$

Results

Overexpression of *OsVP1* and *OsNHX1* in Rice

The rice vacuolar *OsNHX1* (GenBank accession no. AK064004) and *OsVP1* (GenBank accession no. AK099807) were amplified by RT-PCR. After sequencing, the two ORFs were cloned into binary vector pTF102, driven by a CaMV 35S promoter and a NOS terminator (Fig. 1a, b). After selecting transformed rice calli with Basta, we generated ten independent transgenic lines of each construct in "Zhonghua11," a cultivar sensitive to salinity and drought stresses. The corresponding transgenic lines were designated as OsVP1-OE and OsNHX1-OE. All confirmed transgenics displayed normal growth and fertility.

Segregation patterns of herbicide resistance were analyzed in the T_1 generations of 20 independent transgenic events. To gain more information about the function of *OsVP1*, we obtained the *vp1* mutant line (T10221T) from a Tos17-based insertional mutant library of japonica variety "Nipponbare" (Miyao et al. 2003). This contained an insertion of the transposon element in *OsVP1*. RT-PCR was performed to determine the level of expression for *OsVP1* and *OsNHX1* in the overexpressing transgenic lines and *vp1* mutants. In young leaves, two *OsVP1*-OE lines and three *OsNHX1*-OE lines showed high expression by the genes of interests (Fig. 2a, b). This pattern was further verified by subsequent real-time quantitative PCR (Fig. 2c). Compared with the WT, transcripts were much less abundant in the homozygous *vp1* mutant identified from the *Tos17* rice mutant database. Lines with the greatest expression—line 2 from *OsVP1*-OE and line 6 from *OsNHX1*-OE—were selected for further study. Our herbicide resistance assay demonstrated that the selectable marker *bar* segregated at a 3:1 Mendelian ratio for both transgenic lines, indicating that the foreign gene in both transformants was inserted at a single chromosomal locus.

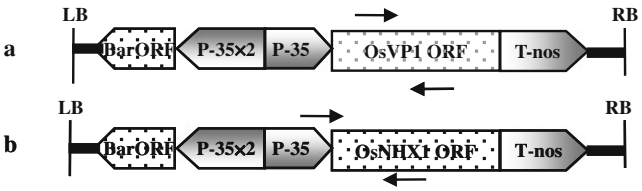

Fig. 1 Constructs for *OsVP1* (**a**) and *OsNHX1* (**b**) overexpression. *Arrows* indicate PCR primers. *LB* and *RB* are left and right border sequences, respectively, of T-DNA. *Bold arrows* show PCR-amplified region for confirming existence of foreign genes in regenerated plants. *P-35* Cauliflower mosaic virus 35S promoter. *Bar* Gene coding for phosphinothricin acetyltransferase, used as selectable marker. *T-NOS* Terminator of nopaline synthase gene

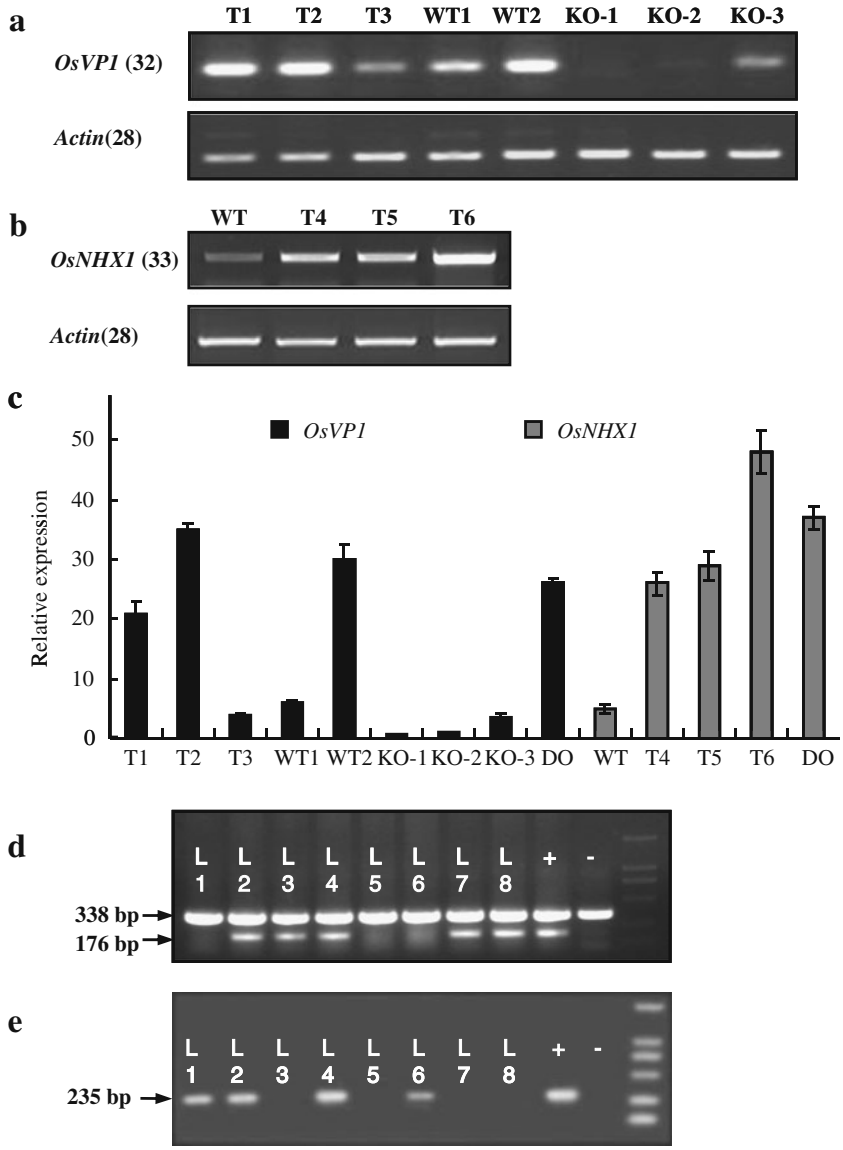

Fig. 2 RT-PCR of *OsVP1*-OE (**a**) and *OsNHX1*-OE (**b**) transgenic plants. *T1*, *T2*, and *T3* are overexpression lines of *OsVP1*. *WT1* and *WT2* are "Zhonghua11" and "Nipponbare," respectively. *KO-1*, *KO-2*, and *KO-3* are *tos17* insertion lines whose background was "Nipponbare." *T4*, *T5*, and *T6* are overexpression lines of *OsNHX1*. **c** Real-time RT-PCR of overexpression lines. **d**, **e** Screening of transgenic seedlings overexpressing both *OsVP1* and *OsNHX1*. **d** PCR of *OsVP1*. Upper band (338 bp) and lower band (176 bp) were amplified from genomic DNA and cDNA using a primer set that covered the region containing the 162-bp intron sequence. "+" and "–" indicate positive controls for *OsVP1*-OE transgenic plant and "Zhonghua 11," respectively. **e** PCR of *OsNHX1*. *L1–L8* are independent lines of *OsVP1*-OE transgenics. The 235-bp product was amplified using primers that covered part of the CaMV 35S promoter and the 5′-end short segment of *OsNHX1*. "+" and "–" indicate positive and negative controls, respectively

Homozygous transgenic T3 plants from *OsVP1*-OE or *OsNHX1*-OE were crossed with each other, and their seeds were then propagated to acquire F_2 seeds. The resultant seedlings were screened via gene-specific PCR for the occurrence of *OsVP1* and *OsNHX1* (Fig. 2d, e). Lines overexpressing both genes did so at higher levels for each compared with the WT (Fig. 2c) and were further examined as double overexpressers.

Effect of *OsVP1* and *OsNHX1* Overexpression on Tolerance to Salinity

To test the effects of double overexpression by both genes on salt tolerance by transgenic rice, we studied 2-week-old seedlings from homozygous lines of *OsVP1* and *OsNHX1* as well as DO plants from F_2. After treatment with 100 mM NaCl for 3 weeks, WT plants showed leaf chlorosis and

gradually died, while plants overexpressing *OsVP1*, *OsNHX1*, or both had enhanced tolerance (Fig. 3a). Respective survival rates for the *OsVP1*-OE, *OsNHX1*-OE, and DO lines were 85.33%, 100%, and 100%, all of which were significantly higher than the 38.67% recorded from the WT (Table 1). Seedling heights, root lengths, and fresh weights were also significantly greater for the transformed lines (Table 1 and Fig. 3b). Finally, Na^+ concentrations in leaves were lower in the transgenics than in the WT (Fig. 3c).

Relative water content is an indicator of leaf water status. When WT plants were grown with 100 mM NaCl for 1 week, their RWC values decreased 12%, whereas no significant differences were found among *OsVP1-OE*, *OsNHX1*-OE, and DO plants when grown under either normal or high-salt conditions (Fig. 3d). Such treatment also induced a greater accumulation of proline in transgenic plants (Table 1). Because proline can potentially lead to osmotic adjustments, it seems that the transgenic plants were able to drive the uptake of additional water by accumulating more soluble substance.

To investigate the degree of membrane peroxidation and changes in photosynthesis, we measured MDA contents and net photosynthetic rates under either normal or high-salt conditions. Whereas exposure to excess NaCl increased MDA values in both transgenic and WT plants, those measured from *OsNHX1*-OE and DO (except *OsVP1* transformants) were significantly lower (Table 1). This suggested that overexpression of *OsNHX1* alleviated salt-induced damage to the membrane system. Under such stress, P_n was significantly diminished in the WT, whereas that rate was maintained at a near-normal level in the transgenics (Table 1). This indicated that overexpression of *OsVP1*, *OsNHX1*, or both could protect the photosynthesis apparatus of rice plants from salt damage.

Effect of *OsVP1* and *OsNHX1* Overexpression on Drought Tolerance

After 2 weeks of water-deficit treatment, the leaves of WT seedlings grown at 20% MWC were wilted (Fig. 4a),

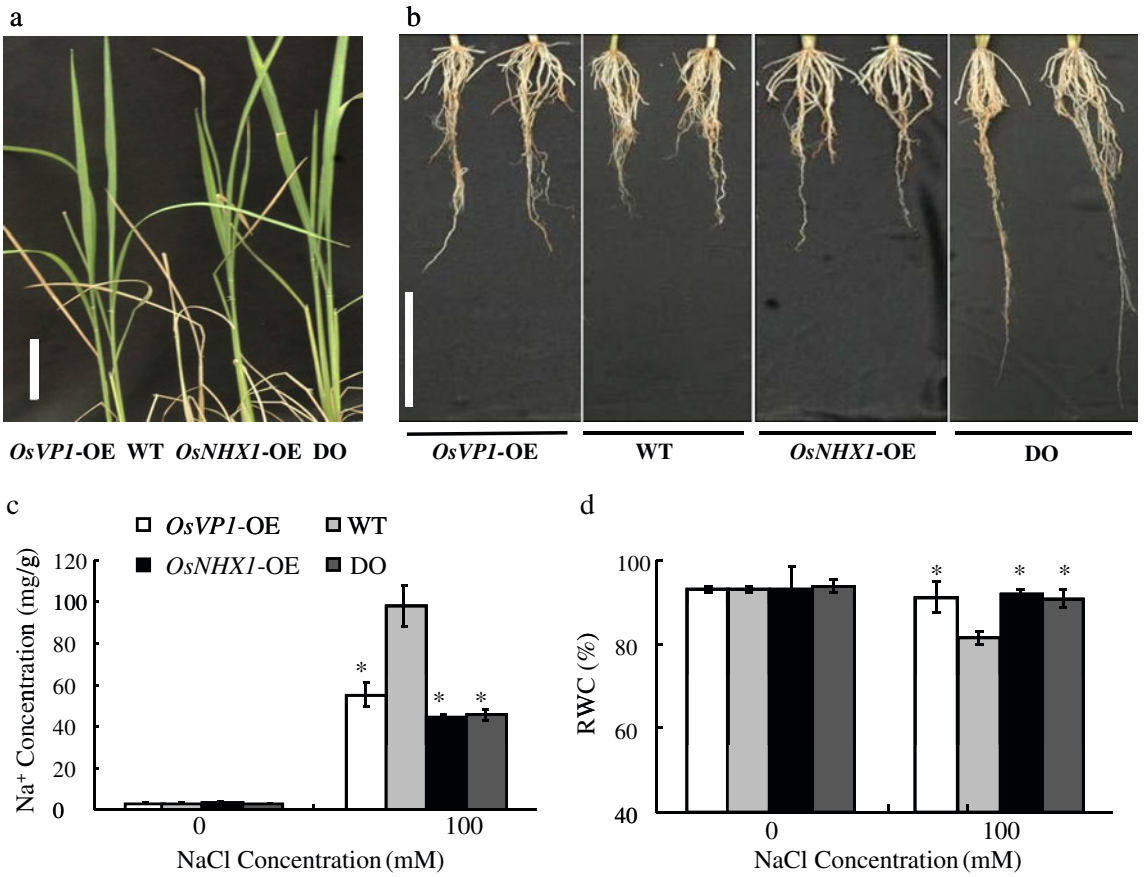

Fig. 3 Growth of *OsVP1*-OE, *OsNHX1*-OE, DO, and WT plants grown in soil and treated with 100 mM NaCl for 25 days. **a** Shoot development from two plants under salt stress. **b** Root growth from plants exposed to 100 mM NaCl. *Bars*, 5 cm. **c** Sodium concentration. *Asterisk* indicates significant difference from WT at $P<0.05$ ($n=3$). **d** RWC in leaves treated with 100 mM NaCl for 7 days. *DO* overexpression of both *OsVP1* and *OsNHX1*

Table 1 Effects of salt stress on transformants and wild-type rice seedlings

	Mock control[a]				NaCl stress[b]			
	WT	*OsVP1*-OE	*OsNHX1*-OE	DO	WT	*OsVP1*-OE	*OsNHX1*-OE	DO
Survival rate (%)[c]	100	100	100	100	38.67±8.25	85.33±14.28[d]	100[d]	100[d]
Height (cm)	43.24±2.33	41.11±5.74	41.83±1.99	41.14±1.13	26.01±3.36	28.46±1.24	26.63±0.77	30.99±2.28[d]
Fresh weight (g)	0.92±0.07	0.87±0.12	0.78±0.19[d]	0.95±0.10	0.32±0.19	0.58±0.09[d]	0.62±0.11[d]	0.61±0.14[d]
MDA ($\mu mol\,g^{-1}$)	4.18±0.24	3.63±0.25[d]	4.54±0.17[d]	4.04±0.32	6.50±0.14	6.43±0.31	5.42±0.38[d]	5.07±0.33[d]
Proline ($\mu g\,g^{-1}$)	37.31±13.20	35.67±9.25	40.18±13.46	38.19±15.26	50.23±10.22	54.32±11.29	60.47±19.20[d]	55.88±16.73
P_n ($\mu mol\ CO_2\ m^{-2}s^{-1}$)	12.45±1.43	13.33±4.45	14.53±2.50	13.78±3.24	7.775±3.48	9.93±1.64	15.55±4.04[d]	14.85±4.53[d]

[a] All data were collected from three replications of five seedlings each and are presented as mean ± SD

[b] Seedlings were treated with 100 mM NaCl for 2 weeks

[c] Survival was assessed with 20 seedlings

[d] Significantly different from the WT at $P<0.05$ ($n=5$)

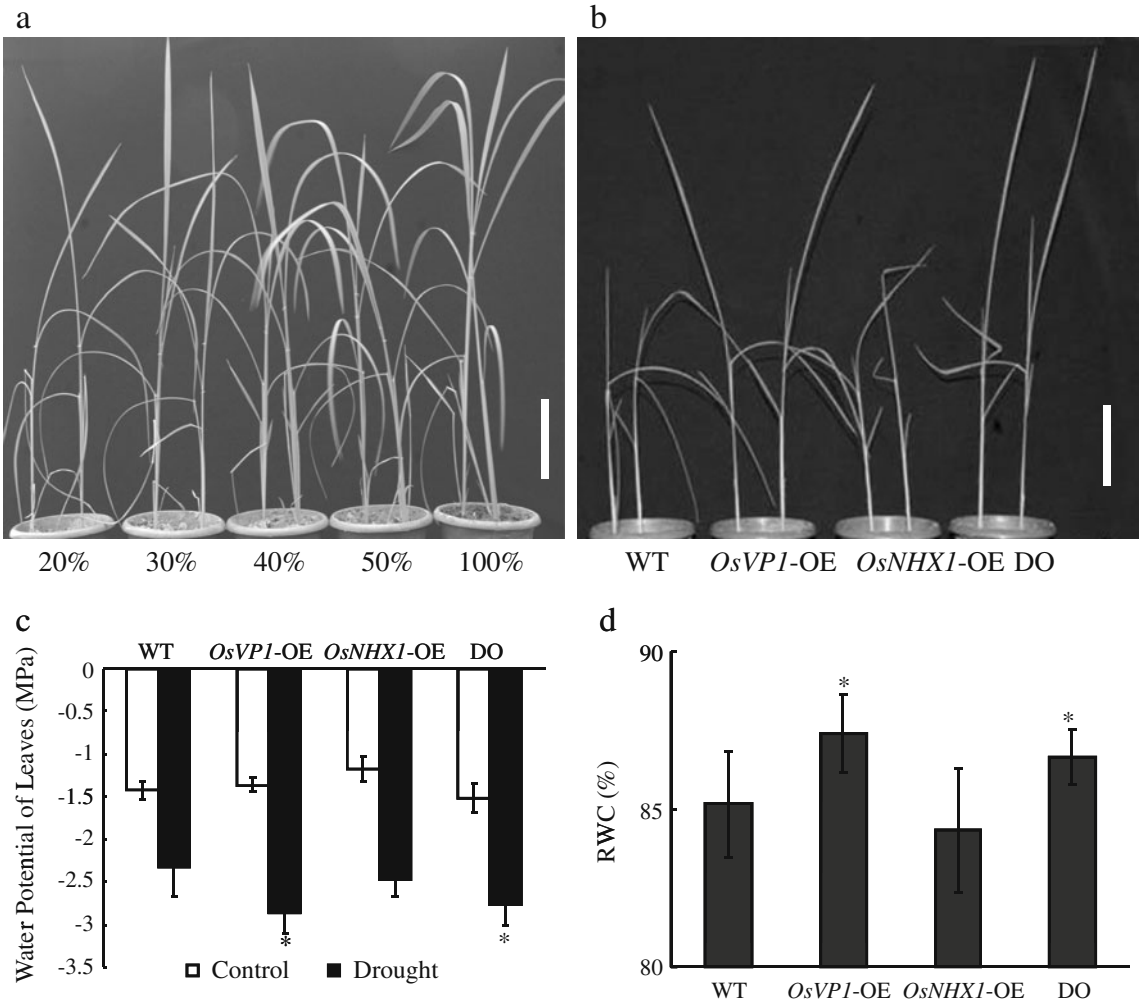

Fig. 4 **a** Seedlings were grown in pots for 14 days under drought conditions, with soil water capacity maintained at 20%, 30%, 40%, 50%, or 100%. **b** Different transgenic lines exposed to 20% maximum water content. *Bars*, 5 cm. **c** Water potential of leaves against drought treatment. **d** RWC after 1 week of water deficit. *Asterisk* indicates significant difference at $P<0.05$ ($n=5$)

whereas *OsVP1*-OE and DO plants exhibited less damage, as defined by their wilting status (Fig. 4b). Further analysis revealed that RWC values were higher in *OsVP1*-OE and DO leaves than in the WT (Fig. 4d). These results suggested that *OsVP1* could enhance drought tolerance in overexpressing transgenic plants.

When plants are exposed to a moisture deficit, leaf water potential decreases, with the extent of that reduction being reflected by their ability to acquire water from their surroundings. Under normal conditions, we found no significant difference in leaf water potential between transgenic and WT plants. However, that parameter was down-regulated in *OsVP1* and DO plants under drought stress (Fig. 4c), suggesting that this osmotic adjustment was triggered by overexpression of *OsVP1*.

Discussion

Salt and drought stresses are major abiotic factors that limit rice productivity. To improve their tolerance to such conditions, we engineered rice plants to overexpress *OsNHX1* and/or *OsVP1*. Compared with the WT, transgenic plants were able to survive under mild stress. Overexpression of *OsNHX1* and *OsVP1* significantly reduced stress-induced damage by eliciting improvements in root growth, water uptake, and leaf water potential, but it had no detrimental impact on plant growth and development (data not shown). "Zhonghua11" proved to be more sensitive to drought and salinity than "Nipponbare" (data not shown), perhaps because expression was lower in the former cultivar. Therefore, this demonstrates that OsVP1 plays an important role in conferring tolerance to drought and salinity.

Studies of many plant species have revealed that *NHX* genes can increase salt tolerance by reducing Na^+ contents in the leaves (Ohta et al. 2002; Brini et al. 2007). Our data also showed that the Na^+ content was similarly reduced in the leaves of *OsNHX1* overexpression lines. This implies that enhanced salt tolerance in transgenic rice might result from having less Na^+ in both the cytoplasm and the leaves. Moreover, we found that plants from those overexpressing lines had lower fresh weights, higher proline contents, and greater P_n values, which may partly explain the balance achieved between the demand and capacity for obtaining additional water.

Simultaneous expression of *SsNHX1* and *AVP1* confers better tolerance to salt stress than does expression by a single *SsNHX1* (Zhao et al. 2006). It is possible that AVP1 activity produces an increased electrochemical gradient to promote the exchange of Na^+/H^+, by which sodium is sequestered into the central vacuole or pre-vacuolar compartments. However, Zhao et al. (2006) have reported no significant difference in drought tolerance between double overexpression lines and *OsVP1* single-gene transformants.

Under stress, plants accumulate more solutes via osmotic adjustment (Zhang et al. 2004). To maintain turgor, cells must keep an osmotic balance between the vacuoles and cytoplasm (Zhang et al. 2004). Here, we observed greater proline accumulations in the cells of *OsVP1*- and *OsNHX1*-overexpressing plants. This action helped in the retention of water within *OsVP1* leaves. The lower leaf water potential (solute potential) recorded for transgenic plants indicated that osmotic regulation mediated by *OsVP1* and *OsNHX1* increased the water potential gradient between leaves and soil, which then served as a force to drive water uptake.

We found that transgenic rice incurred less serious Na^+ toxicity under salt stress, perhaps due to sequestration in the vacuoles that protected the cells. Alternatively, the alleviation of toxic effects from Na^+ may partly be attributed to the decrease in oxidative damage via MDA. Enhanced activity during the imposition of oxidative-stress tolerance is closely associated with the reduced generation of MDA in transformants (Allakhverdiev et al. 1999, 2000). The high rate of photosynthesis calculated from our transgenic rice also confirmed that those plants had less damage from salt stress. This may have been related to an increase in Na^+ sequestration, which protects the photosynthesis apparatus.

In many plant species, the status of the root system is closely associated with tolerance to drought (Mittova et al. 2004). Overexpression of *AVP1* in both *Arabidopsis* and tomato leads to increased root growth under a water deficit (Gaxiola et al. 2001; Park et al. 2005). This up-regulation also induces a greater accumulation of solutes in the leaves of transgenic *Arabidopsis*, but not in transgenic tomato. In our study, salt stress promoted the development of more robust root systems in DO and *OsVP1* transformants. This is consistent with those results reported above for *AVP1*-overexpressing *Arabidopsis*, suggesting that for rice, high salinity induces tonoplast Na^+/H^+ antiporter activity in the roots but not in the shoots. Increased tolerance to salt stress in our transgenic plants may have been a result of overexpression by foreign genes in the roots. This promoted the formation of a root system that was better adapted to such stress. Moreover, transporting more Na^+ into the root vacuoles would have caused the Na^+ content to be lower in the cytosol. Similar findings have been noted with *AtNHX1*-overexpressing tomato and *Brasssica napus*, plants in which improved root growth leads to enhanced cellular Na^+ exclusion (Apse et al. 1999).

In summary, our experiments demonstrate that *OsNHX1* and *OsVP1* have great potential for use in improving stress tolerance in rice, perhaps through the activity of multiple mechanisms. Therefore, screening for the highest level of

expression by either *OsVP1* or *OsNHX1* in rice germplasm could be a useful tool for breeding programs.

Acknowledgments This work was supported by the National High Technology Research and Development Program (2007AA021403), the Specialized Research Fund for the Doctoral Program of China Higher Education (grant no. 20070335081), and the Research Foundation for Talented Scholars in China Three Gorges University (KJ2009B047).

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